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Aerobic exercise modulation of mental stress-induced responses in cultured endothelial progenitor cells from healthy and metabolic syndrome subjects

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ABSTRACT

Aim: Numerous studies have demonstrated that exercise acutely prevents the reduction in flow-mediated dilation induced by mental stress in subjects with metabolic syndrome (MetS). However, it is unknown whether a similar effect occurs in endothelial progenitors cells (EPCs). This study investigated whether exercise protects from the deleterious effect of mental stress on cultured EPCs in healthy subjects and those with MetS.
Main methods: Ten healthy subjects (aged 31 ± 2) and ten subjects with MetS (aged 36 ± 2) were enrolled. Subjects underwent a mental stress test, followed immediately by either 40 min of leg cycling or rest across two randomized sessions: mental stress + non-exercise control (MS) and mental stress + exercise (MS + EXE). The Stroop Color-Word Test was used to elicit mental stress. Blood samples were drawn at baseline and following sessions to isolate mononuclear cells. These cells were cultured in fibronectin-coated plates for seven days, and EPCs were identified by immunofluorescence ($acLDL^+$/ UEA-I $Lectin^+$).
Key findings: All subjects presented similar increases in mean blood pressure and heart rate during the mental stress test ($P < 0.01$) in both the MS and MS + EXE sessions. Number of EPCs was not different between groups at baseline in both sessions ($P > 0.05$). The EPC response to MS and MS + EXE was increased in healthy subjects, whereas it was decreased in subjects with MetS ($P < 0.04$). In healthy subjects, the EPC response to MS + EXE was greater than the response to MS alone ($P = 0.03$).
Significance: An exercise session increased EPCs in healthy subjects but did not prevent the EPC reduction induced by mental stress among subjects with MetS.

Introduction

Metabolic syndrome (MetS) is a constellation of metabolic risk factors, including high blood pressure, hyperglycemia, dyslipidemia and abdominal obesity. When these risk factors present together, the probability for future cardiovascular problems becomes greater than that of any one factor alone [1]. Subjects with MetS have a two-fold increased risk for heart attack or stroke, and a five-fold increased risk for developing diabetes when compared with individuals who do not have metabolic syndrome [1].

MetS is also associated with endothelial dysfunction, as evidenced by reduced flow-mediated dilation [2,3]. Endothelial dysfunction is a systemic pathological state characterized by an imbalance between mediators of vasodilation and vasoconstriction produced by (or acting on) the endothelium [4]. These alterations can increase both inflammatory markers and the production of reactive oxygen species from the endothelium, contributing to atherogenesis [5].

Endothelial repair can occur by the proliferation of surrounding mature endothelial cells [6]. However, mature endothelial cells have a low proliferative potential, and their capacity to replace damaged endothelium is limited [6]. Therefore, endothelial repair depends on other cell types. Accumulating evidence indicates that the peripheral blood of adults contains a unique subtype of circulating endothelial progenitor cells (EPCs) with similar properties to embryonic angioblasts [7,8]. Recruitment of EPCs requires a coordinated sequence of signaling events, including chemoattraction, migration and adhesion. Jialal et al. [9] demonstrated that the mobilization and migration of EPCs is usually impaired in subjects with MetS. However, it is unclear whether the adhesion capacity of EPCs is preserved in MetS.

In addition to MetS, psychosocial disorders such as mental stress represent important risk factors for cardiovascular morbidity and mortality [10]. Chronic exposure to stress is usually associated with endothelial dysfunction [11–13] and the depletion of circulating EPCs

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in healthy subjects [14]. This deleterious process on endothelial function begins during mental stress, can last up to 90 min [11], and its magnitude is considered clinically meaningful [15]. Consequently, preventing this process might be crucial for cardiovascular health protection, particularly among subjects with a cluster of risk factors, such as those with MetS [16,17].

Lower-limb aerobic exercise acutely improves endothelial function [18,19] and prevents a reduction in the brachial artery flow-mediated dilation induced by mental stress among subjects with cardiometabolic risk factors [3]. This type of exercise is also capable of modulating hemodynamic responses (i.e., blood pressure, stroke volume and cardiac output) and mental stress in healthy and pre-hypertensive subjects [20,21]. As such, it is expected that a single bout of exercise would protect the number and function of EPCs from the deleterious effects of mental stress; however, this hypothesis currently remains untested.

This study aimed to investigate the effect of mental stress followed by a bout of exercise on cultured EPCs in both healthy subjects and those with MetS. We hypothesized that exercise prevents a reduction in the EPC response to mental stress in subjects with MetS.

Materials and methods

Ethics statement

All experimental procedures and protocols were consistent with the principles of the Declaration of Helsinki, and were approved by the Institutional Review Boards of the Fluminense Federal University (CAAE 006.0.258.000-10). All subjects provided written informed consent before the study and their rights were protected.

Subjects

Subjects were recruited through advertisements at the University and in local newspapers. Twenty subjects were enrolled, ten subjects with early MetS (MetS group, age: 36 ± 2) and ten healthy subjects (controls) with none of the five criteria for MetS (Healthy group, age: 31 ± 2). The MetS group presented at least three of the following five criteria defined by the American Heart Association [17]: waist circumference >90 cm (men) or >80 cm (women); systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; fasting blood glucose ≥100 mg.dL^{-1}; plasma triglycerides ≥150 mg.dL^{-1}; high-density lipoprotein cholesterol (HDL-c) <40 mg.dL^{-1} (men) or <50 mg.dL^{-1} (women). Other inclusion criteria included the absence of any diagnosed disease, no recent infection, no medication, non-smoker, women with regular menstrual cycles, and maintaining a sedentary lifestyle (not engaged in exercise activities lasting ≥30 min for at least three times weekly during the last three months). Women who had regular menstrual cycles were evaluated during the early follicular phase (up to 5 days after the onset of menstruation). The eligibility requirements were verified through a clinical history assessment, physical examination, blood pressure measurement, biochemical blood analyses, resting electrocardiogram, and peak cardiopulmonary exercise testing.

Biochemical blood analyses

Blood was drawn in the morning from an anterior cubital vein following a 12 h fast. Cholesterol and its subfractions [HDL-c and low-density lipoprotein (LDL-c)], as well as circulating triglycerides and glucose were determined using enzymatic colorimetric methods. Total leukocyte count was measured by an electronic counter, the HST-302 N system (Sysmex Corporation, Kobe, Hyōgo Prefecture, Japan).

Clinical evaluation

A physician conducted the evaluation, which included a clinical history assessment and a resting electrocardiogram (CardioCare 2000, Bionet, Tustin, CA, USA). With the participant in an upright sitting position, resting blood pressure measurements were performed twice, one on each arm, on two separate days. Recordings were made under quiet and comfortable (approximately 24 °C) laboratory conditions. An appropriately sized cuff (cuff bladder encircling at least 80% of the arm) was used.

Physical examination

Weight and height were measured using a medical beam balance (Welmy, Santa Bárbara d'Oeste, SP, Brazil). Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m). Waist circumference was considered as the midpoint between the iliac crest and the last floating rib (XII rib).

Cardiopulmonary exercise testing

Subjects underwent a standard cardiopulmonary exercise test, performed until exhaustion on a cycle ergometer (CG400 model, Inbrasport, Porto Alegre, RS, Brazil) [3]. The protocol was individualized according to predicted maximal exercise capacity. Subjects were verbally encouraged to exercise until exhaustion to reach volitional fatigue at approximately 10 min of testing. Ventilation, oxygen uptake, and carbon dioxide output were measured with each breath (CPX Ultima Gas Exchange System, Medgraphics Corp, St Paul, MN, USA). An electrocardiogram was monitored through 12 leads (Welch Allyn CardioPerfect Workstation, Welch Allyn, Skaneateles Falls, NY, USA), and perceived exertion was assessed every minute using the 0–10 Borg scale. Breath-by-breath ventilation and expired gas data were averaged every 20 s to identify the peak oxygen consumption (VO_2peak), which was considered the highest value of oxygen uptake recorded during exercise. The ventilatory threshold was identified by a combination of the following methods: 1) Inflection of ventilation vs. time curve and 2) A consistent increase in the ventilatory equivalent of oxygen (VE/VO_2) without a concomitant increase in the ventilatory equivalent of carbon dioxide (VE/VCO_2).

Experimental protocol

On two separate days, at least two days apart, subjects from both the healthy and MetS groups underwent a mental stress + non-exercise control session (MS) and a mental stress + exercise session (MS + EXE) in random order (Fig. 1). Both sessions began with a blood draw for EPC isolation. Then, a mental stress test was induced, and the subjects underwent either a non-exercise control period or a bout of exercise. A blood draw was repeated after the control period or bout of exercise. During the MS session, subjects sat still on the cycler ergometer for the same period of time as the MS + EXE session. These experimental sessions were always conducted at the same time of day after a 1-h fast. Participants were also given standard feeding orientations for the previous day and abstained from caffeine and alcohol consumption, as well as physical exercise for at least 48 h.

Mental stress test

Mental stress was elicited over a 3-min period using an adapted, computerized version of the Stroop Color-Word Test [22]. The test consisted of a slideshow projected on the ceiling in front of the subjects. The slides changed every 2 s. Auditory conflicts were continuously delivered via earphones using a standardized audio clip integrated into the slideshow. Perceived stress level was recorded after each test

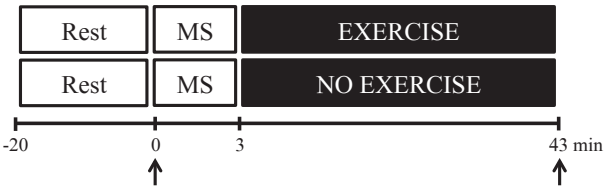

Fig. 1. Protocol design. Small arrows indicate blood draws. MS, mental stress.

using a 5-point scale as follows: 0 (not stressful), 1 (somewhat stressful), 2 (stressful), 3 (very stressful) and 4 (extremely stressful) [23].

Exercise bout

A continuous submaximal bout of exercise was performed for 40 min on a cycle ergometer (CG400 model, Inbrasport, Porto Alegre, RS, Brazil) at an intensity corresponding to 80% of the ventilatory threshold, which was identified during the previous cardiopulmonary exercise test. The exercise bout was preceded by a 5 min warm-up of pedaling at 30 W, followed by 5 min of recovery pedaling at 30 W. Breath-by-breath ventilation and expired gas were recorded throughout the exercise bout by a digital metabolic analyzer (CPX Ultima Gas Exchange System, Medgraphics Corp, St Paul, MN, USA), which was linked to a computer for data recording and off-line analysis.

Endothelial progenitor cell evaluation

EPCs were analyzed by immunofluorescence. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation (Histopaque 1077, Sigma-Aldrich, St. Louis, MO, USA) from 25 mL of blood according to the instructions provided by the manufacturers. The plasma was stored in a −20 °C freezer until use in culture techniques.

Cell culture and immunofluorescence

After isolation, 1×10^6 cells from each subject were plated on a 24-well fibronectin-coated plate (5 μg/mL; Sigma) in endothelial growth medium type 2 (EGM-2; Lonza, Visp, Switzerland) containing 2% fetal bovine serum, hydrocortisone, ascorbic acid, gentamicin-amphotericin, heparin and growth factors (hFGF-B, hEGF, VEGF, R_3-IGF-1). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C for four days. On the fourth day, non-adherent cells were removed during the first medium change. Next, the subsequent EGM-2 changes were performed every 48 h, and 10% of plasma from the respective time point was added to the culture to maintain the mental stress and exercise/non-exercise conditions. The adherent cells were maintained under standard culture conditions for more three days.

On the seventh day of culture, the adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil-acLDL; Life Technology, Carlsbad, CA, USA) for 4 h at 37 °C. The cells were then fixed with 4% paraformaldehyde and counter stained with FITC conjugated to *Ulex Europaeus agglutinin* I Lectin (FITC UEA-I Lectin, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. All the cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Double-positive cells ($acLDL^+$ /UEA-I $Lectin^+$) were considered EPCs, and the quantity of these cells was expressed as percentage of the total number of adherent cells. Two independent investigators conducted a manual quantification in four random fields of each well, and their results averaged. We used the image acquisition system of a Leica microscope at a magnification of 200× (DMI 3000 model, Leica DFC 310 FX camera) and the Leica Application Suite V3 software (Leica Microsystems, 35578 Wetzlar, Hesse, Germany). A negative control was used to analyze cellular auto-fluorescence.

Statistical analysis

Power analysis of the study's main endpoint (i.e., response of EPCs to MS and MS + EXE sessions analyzed by two-way ANOVA) has shown a statistical power of 0.99 for any of the main effects or interactions. Data distribution was verified through the Shapiro–Wilk test, and homogeneity of variance by the Levene's test. A two-tailed unpaired Student's t test was performed to identify significant between-group differences in all normally distributed demographic and clinical variables. When distributional assumption of normality was not met, the statistical inference was obtained using the Mann–Whitney U test. The Chi-square test was used to analyze categorical variables. A three-way ANOVA was used to compare cellular and hemodynamic variables [factors: group (healthy vs. MetS), session (MS vs. MS + EXE) and time (pre vs. post)], while a two-way ANOVA [factors: group (healthy vs. MetS) and session (MS vs. MS + EXE)] was used to compare the response (i.e., absolute change = post − pre) of EPCs to MS or MS + EXE between groups. ANOVAs were followed by the Fisher post hoc test in the event of a significant group, moment and/or interaction effect. The data are presented as the mean ± standard error of the mean (SE) or median ± interquartile differences, when appropriate. Significance was accepted at the 0.05 level.

Results

The anthropometric, clinical, and biochemical profiles of healthy subjects and those with MetS are presented in Table 1. As expected, body mass, body mass index (BMI), body fat, waist circumference, blood pressure, lipid profile and glucose profile were significantly different between the healthy and MetS groups. There were no differences between the groups regarding gender, age and total leukocyte count.

Table 2 demonstrates the effect of mental stress on mean blood pressure and heart rate. We verified that mean blood pressure and heart rate increased similarly during mental stress in healthy subjects

Table 1
Selected subject characteristics.

Variable	Healthy	MetS	*P* value
No.	10	10	–
Age, yr	31 ± 2	36 ± 2	0.16
Sex, M/W	8/2	9/1	0.53
Body mass, kg	68 ± 6	98 ± 4	<0.01
BMI, kg/m^2	23 ± 1	32 ± 1	<0.01
Body fat, %	25 ± 2	36 ± 3	<0.01
Waist circumference, cm	79.2 ± 3.0	104.6 ± 3.3	<0.01
Systolic BP, mmHg	116 ± 2	128 ± 5	0.02
Diastolic BP, mmHg	77 ± 2	85 ± 3	0.02
VO_{2peak}, $mL/Kg.min^{-1}$	32 ± 3	24 ± 3	0.05
Total cholesterol, mg/dL	176 ± 7	211 ± 11	0.02
LDL-cholesterol, mg/dL	108 ± 8	136 ± 12	0.06
HDL-cholesterol, mg/dL	54 ± 4	41 ± 3	<0.01
Triglycerides, mg/dL	66 ± 6	170 ± 16	<0.01
Glucose, mg/dL	85 ± 2	101 ± 4	<0.01
Total leukocyte count, $10^3/mm^3$	5.80 ± 0.45	7.03 ± 0.64	0.16

Values are the means ± SE. M, men; W, women; BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 2
Response of mean blood pressure and heart rate to mental stress.

Sessions		Mean arterial pressure (mmHg)		Heart rate (bpm)	
		Healthy	MetS	Healthy	MetS
MS	Pre	94 ± 4	97 ± 4	60 ± 3	66 ± 3
	Post	108 ± 7*	108 ± 6*	72 ± 5*	76 ± 5*
MS + EXE	Pre	96 ± 4	101 ± 4	58 ± 3	69 ± 2
	Post	108 ± 5*	114 ± 5*	72 ± 5*	82 ± 4*

Values are the means ± SE. (*) $P < 0.01$ *vs* pre. MS, mental stress + non-exercise control session; MS + EXE, mental stress + exercise session.

and those with MetS ($P < 0.01$), in both the MS and MS + EXE sessions. No changes were observed in subjects' perceived stress between groups ($P = 0.16$) or between sessions ($P = 0.44$).

Primary EPC cultures in EGM-2 showed spindle-shaped cells after seven days on 24-well fibronectin-coated plates. Phenotype characteristics of EPC cultures were measured using immunofluorescence microscopy (Fig. 2). It was analyzed morphology (Fig. 2A), acLDL uptake (Fig. 2C) and UEA-I binding (Fig. 2D). EPCs were considered as double-positive cells (acLDL+ /UEA-I Lectin+; Fig. 2E).

Fig. 3 shows cultured EPCs before and after MS or MS + EXE in healthy subjects and those with MetS. In MS and MS + EXE sessions, no differences were observed between groups at baseline ($P > 0.05$). However, the number of EPCs decreased only in subjects with MetS after MS ($P = 0.03$). After MS + EXE, the number of EPCs increased in healthy subjects ($P < 0.01$), whereas no differences were observed in subjects with MetS ($P = 0.11$). The number of cultured EPCs was similar between groups after MS ($P = 0.71$) or MS + EXE ($P = 0.85$).

The response of EPCs (Fig. 4) to both MS and MS + EXE was positive (increased) in healthy subjects, whereas it was negative (decreased) in subjects with MetS ($P < 0.04$). In healthy subjects, EPC response to MS + EXE was greater than the response to MS by itself ($P < 0.01$).

Discussion

The present study investigated the potential protective effect of exercise from the deleterious effect of mental stress on the number of

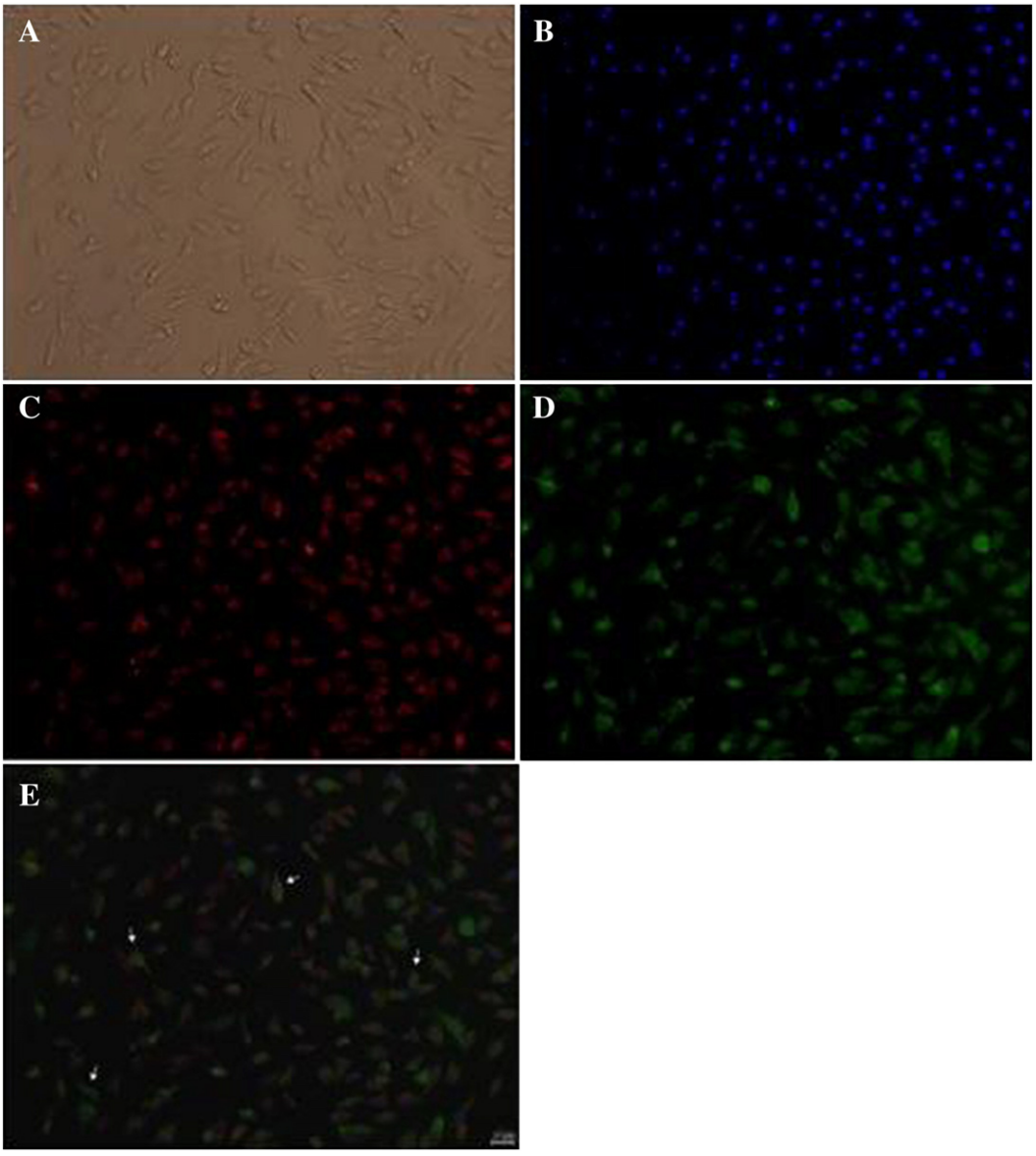

Fig. 2. Characterization of endothelial progenitor cells derived from peripheral blood by immunofluorescence. A, cells in phase contrast; B, DAPI nuclear counterstain; C, cells positive for acLDL uptake; D, cells positive for UEA-I Lectin; E, double-positive cells (arrow). Scale bar: 20 μm. DAPI, 4′, 6-diamidino-2-phenylindole; acLDL, acetylated low-density lipoprotein; UEA-I, Ulex europaeus agglutinin-I.

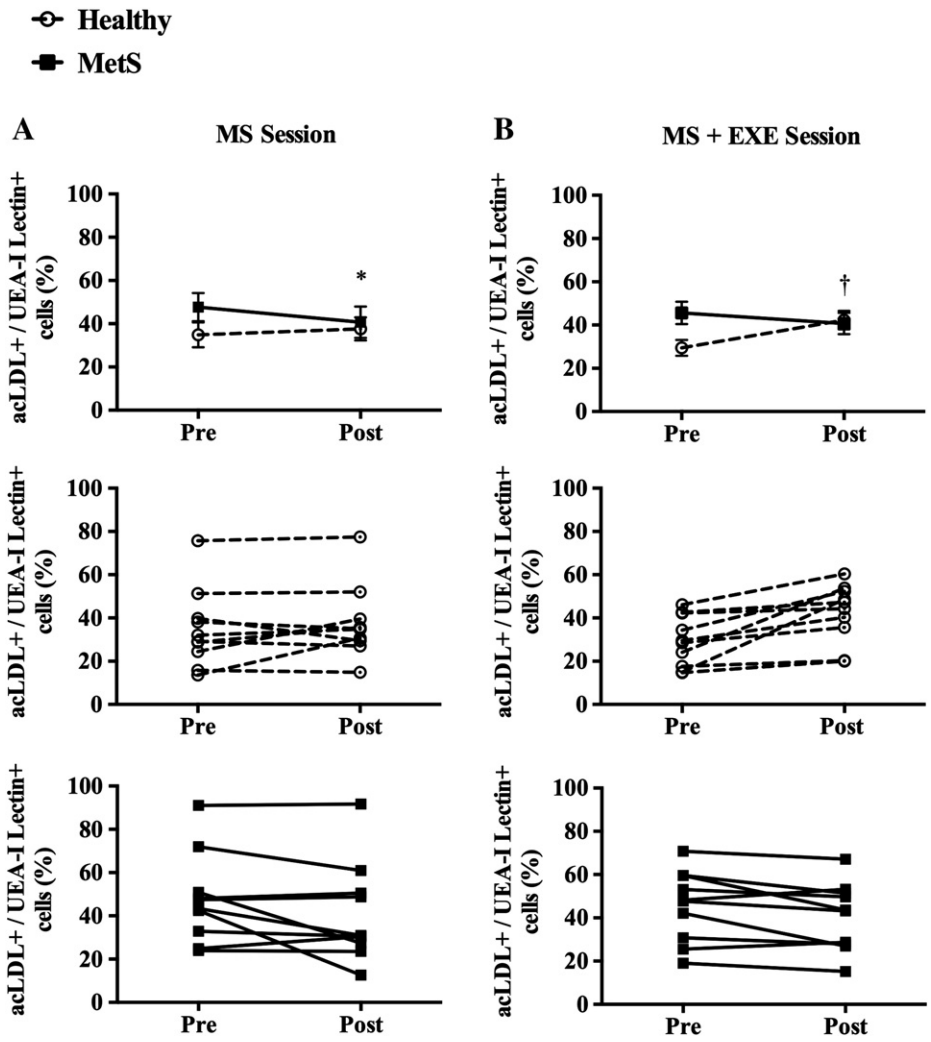

Fig. 3. Double-positive cells (acLDL + /UEA-I Lectin +) before and after a mental stress + non-exercise control session (A) or a mental stress + exercise session (B) in healthy subjects and those with MetS. (*) $P = 0.03$ *vs.* pre in MetS. (†) $P < 0.01$ *vs.* pre in healthy subjects. Healthy, healthy subjects; MetS, subjects with metabolic syndrome; MS, mental stress + non-exercise control session; MS + EXE, mental stress + exercise session.

cultured EPCs in healthy subjects and those with MetS. Novel findings of this study were two-fold: 1) A single bout of exercise increased EPC number after the mental stress test in healthy subjects; and 2) A single bout of exercise was unable to prevent the reduction of EPCs in subjects with MetS.

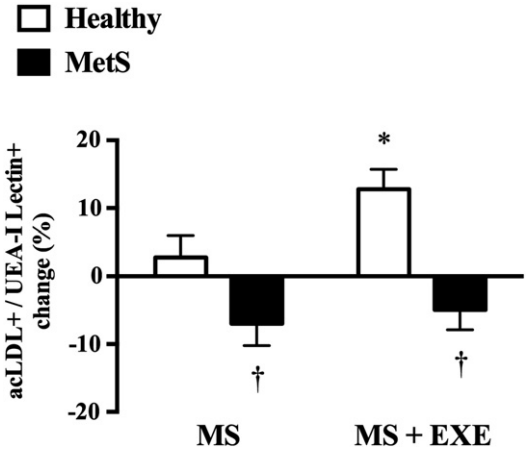

Fig. 4. Response of double positive cells (acLDL +/UEA-I Lectin +) to mental stress + non-exercise control session (MS) or mental stress and exercise (MS + EXE) in healthy subjects and those with MetS. (*) $P = 0.03$ *vs.* MS session; (†) $P < 0.04$ *vs.* healthy subjects. Healthy, healthy subjects; MetS, subjects with metabolic syndrome; MS, mental stress + non-exercise control session; MS + EXE, mental stress + exercise session.

Under physiological conditions, EPCs play an important role in vascular damage repair, and it has been suggested that a decreased number of EPCs is associated with an increased prevalence of subclinical atherosclerosis [24]. The functional properties of EPCs, as evidenced by in vitro cultivation, may be of equal or potentially greater importance than quantitative alterations of peripheral blood [25]. Studies have shown that rats with MetS have decreased EPC concentrations, colony-forming units, a lowered proliferative capacity and a high apoptosis rate in cultured cells [26]. In humans, subjects with MetS present with a decreased number of colony-forming units and a reduced capacity to form tubules [9]; the present study showed no differences in the number of cultured EPCs between healthy subjects and those with MetS at baseline conditions. However, it is important to consider some particular characteristics of these studies before interpreting their results. Most studies regarding MetS recruited subjects undergoing pharmacological treatment or those with overt diseases, which can bias the number or functionality of EPCs. To our knowledge, this is the first study that enrolled only drug-naïve MetS subjects without comorbidities, indicating that they are in an early stage of MetS. Considering these current data alongside previous studies [27], we suggest that these subjects with MetS present with subclinical alterations, which may become more evident during physiological challenges such as exercise or mental stress.

Mental stress tests have been extensively used to evaluate endothelial function in distinct groups with cardiovascular risk. Studies have shown that mild psychological stress in healthy middle-aged subjects is associated with impaired endothelial function, as reflected by decreased flow-mediated dilation [28] and increased carotid intima-media thickness [29]. Our study indicated that a mental stress test was able to similarly stress both healthy subjects and those with MetS, as evidenced by enhanced mean blood pressure and heart rate during the test. Moreover, subjects with MetS, even in early stages, already present with an impaired number of EPCs in culture after mental stress, which correlates with reduced EPC functionality and may contribute to diminished potential for endothelial repair. Other studies [14,30] corroborate our findings, which demonstrate that patients with cardiovascular diseases and a high depression score present with lowered numbers of CD34+/VEGFR2+ EPCs.

Mechanisms underlying this process are still unclear. Mental stress can predispose individuals to inflammation by increasing sympathetic activity, catecholamine release and cortisol secretion [31]. Moreover, it is known that subjects with cardiometabolic diseases, such as MetS, present with reduced levels of anti-oxidative enzymes and increased oxidative stress [32] when compared to healthy subjects. Considering that both inflammation and oxidative stress are able to negatively modulate EPCs [33], the association between mental stress and MetS could lead to reduced EPC proliferative potential or adherence, and increased susceptibility to apoptosis.

The current study demonstrated that exercise increased the number of cultured EPCs in response to mental stress in healthy subjects. Previous studies corroborated our data, which demonstrated that exercise acutely stimulates EPC mobilization from bone marrow to peripheral blood in healthy subjects [34–36]. As a possible mechanism, the authors identified nitric oxide (NO) bioavailability [35]. In fact, it is well-established that augmentation of shear stress induced by exercise through greater anterograde, lesser retrograde, or both types of shear stress increase the expression of endothelial nitric oxide (NO) synthase [37,38].

However, a single bout of exercise was insufficient to prevent a reduction in cultured EPCs induced by mental stress in subjects with MetS. Our group recently published that aerobic exercise acutely prevents a reduction in the flow-mediated dilation induced by mental stress among subjects with MetS [3]. Although controversial, these data may be explained by certain factors.

Acutely, exercise induces a transient inflammatory such as interleukin-6, tumor necrosis factor-α, C-reactive protein [39] and oxidative response [40] in subjects at increased cardiometabolic risk [41]. In contrast, physical training develops over time adaptive compensatory mechanisms to the acute stress of exercise bouts [42], such as reductions in the basal levels of pro-inflammatory cytokines and initiation of antioxidant and anti-inflammatory mediator expression in the vascular wall [41]. Considering that inflammation and oxidative stress are increased in subjects with MetS [39,40], primarily during mental stress, it is suggested that anti-inflammatory and anti-oxidant factors released after training may modulate the EPC response to mental stress in this group of subjects and, consequently, diminish the risk of cardiovascular events [43].

There are some technical issues regarding this study that should be mentioned. First, despite not having a standardized marker to describe, characterize or quantify circulating EPCs, it is well-established that acLDL+/UEA-I Lectin+ cells are capable of being incorporated into vessels undergoing repair and angiogenesis [44]. Second, there was no specific quantitative measurement of EPCs shortly after the sessions. Cell culture is an indirect technique to functional analyses, but also can be used to quantitative evaluation. In order to keep the stimulus of mental stress or exercise/non-exercise sessions in cultured cells during a seven-day culture, the medium was supplemented with 10% plasma of respective session in each medium change. Third, there are few women in either group. Although it might represent a threat to the external validity of the study, an analysis in which women were not included did not alter the results.

Conclusion

In summary, despite increasing the number of cultured EPCs after mental stress in healthy subjects, a single bout of aerobic exercise did not prevent a mental stress-induced reduction in cultured EPCs among subjects with MetS.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgements

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